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of Prostate Cancer

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13. ABSTRACT (Maximum 200 Words)

Radioimmunolocalization of prostate cancer with radiolabeled antibodies is widely used in a clinic but radioimmunotherapy (RIT) fails to produce objective responses. Physiological barriers to the delivery of macromolecules to solid tumors are usually blamed for these failures. These studies are designed to improve the outcome of RIT in prostate adenocarcinoma by the inclusion of the antigen-independent peptides in the RIT protocol. To date two peptides able to modify vascular permeability were tested. Cytotoxicity studies indicate dose-dependent changes in cell metabolic activities after treatment with the C5aAP peptide; whereas peptide able to interact with a formyl peptide receptor-like 1 (FPRL1) does not seem to have any effect on the growth of these cells in vitro. In vivo results indicate that both peptides significantly augment RIT with ^{131}ICC49. Three xenografts were tested to date: LNCaP, PC3 and DU145. These xenografts do not show differences in the growth pattern between the untreated tumors and peptide only-treated tumors, but there is a considerable delay in the tumor growth when peptides are combined with ^{131}ICC49. The mechanism of this effect is more complicated than observed for LS174T tumors evaluated in the pilot studies and appears to vary depending on the tumor model and the peptide. The pattern of dependence on to key two factors emerged from these studies: (1) the improved vascular permeability and (2) the generation of reactive oxygen species.

14. SUBJECT TERMS
Prostate cancer, radioimmunotherapy, antibodies, vascular permeability, C5a agonists

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Table of Contents

Role of polymorphonuclear leukocytes, nitric oxide synthase, and cyclooxygenase in vascular permeability changes induced by C5a agonist peptides. Takashi Kurizaki, Michio Abe, Sam D. Sanderson, Charles A. Enke, Janina Baranowska-Kortylewicz. ***Mol. Cancer Ther.*** 2004; 3(1):1–7.

Introduction

The efficacy of radioimmunotherapy (RIT) of solid tumors depends on a number of factors related to the characteristics of antibody, radionuclide, and the tumor physiology such as the tumor heterogeneity, its size, the antigen distribution, radiosensitivity, microvessel perfusion, and vascular permeability. It is apparent that a success of radioimmunotherapy in solid tumors will require a combination of therapeutic strategies.

One widely studied approach involves the use of systematically administered vasoactive agents, such as angiotensin II (Kinuya et al., 2000), interleukin-2 (Hornick et al., 1999), alpha v beta 3 integrin receptor antagonist (Burke et al., 2000), and the C5a agonist peptides (Kurizaki et al, 2002). The complement derived inflammatory mediator C5a evokes broad anaphylactic and chemotactic responses, including increased vascular permeability, changes in adhesiveness, smooth muscle contraction, and chemotactic migration of a number of cells. These biological activities are mediated through its binding to the C5a receptor (C5aR; CD88). C5a-derived small agonist peptides (C5aAP) from C-terminal region behave as full agonist, but with reduced potency. One analog, YSFKPMPLaR, expressed between 2 and 10% of full C5a activity in increasing vascular permeability and it was stable in the presence of mouse and human serum carboxypeptidases. Our studies showed that combination RIT with C5aAP (YSFKPMPLaR) resulted in two- to five-fold better LS174T xenografts responses than RIT alone. This therapeutic improvement in colorectal adenocarcinoma model was primarily attributed to the agonist-induced increase in the tumor vascular permeability (Kurizaki et al., 2002). In the current study, the effects of C5aAP and other peptides able to change vascular permeability of tumor blood vessels of radioimmunotherapy of prostate cancer are evaluated.

<u>Summary of the Statement of Work Progress</u>

These studies were designed to evaluate the effect of antigen-independent peptides on the outcome of RIT in experimental models of the human prostate adenocarcinoma. In Objective 1: (determination of the uptake of ^{125}I-labeled B72.3 monoclonal antibody in three human prostate adenocarcinoma models: PC-3, DU-145 and LNCaP in athymic mice) the following specific tasks are included:

1. In vitro culture of PC-3, DU-145, LNCaP for implantation into mice: *months 1 – 36*
   In progress
   This is an ongoing task. Because TAG-72 antigen is only expressed in tumors grown in vivo, a constant supply of large number of cultured cells for implantation into mice is needed.
2. Radiolabeling of B72.3 with iodine-131 and iodine-125 *months 1 – 36*
   In progress
   This is also an ongoing task. All biodistribution studies are done with iodine-125-labeled antibodies. For therapy studies we are using iodine-131 as the radioisotope.
3. Immunohistochemistry to determine TAG-72 expression *months 1 –6*
   Completed: reported in the progress for the first year of funding.
   In addition to biodistribution studies, we have also evaluated the expression of the antigen in frozen tumors collected during the necropsy.

For Objective 2 (to measure the effect of C5aAP on the uptake of 125I-labeled B72.3 monoclonal antibody in a prostate cancer model selected in Aim 1 for the expression of TAG-72 antigen) the following specific tasks were planned:

1. Synthesis of peptides   *months 12 –36*
   In progress
   This is an ongoing task in years 2 and 3 of this project. Initial studies were done with the previously used C5aAP analog. Currently a new peptide is evaluated in search of a more response-selective derivatives.

2. Biodistribution of ^{125}IB72.3 in tumor-bearing mice   *months 1 –36*
   In progress
   The biodistribution of the antibody is established in the selected tumor models. We are still also evaluating the effects of peptides dose and dosing schedule on the fate of radiolabeled antibodies. This is be done either via a terminal procedures, i.e., necropsy of tumor-bearing animals as well as via an MRI studies.

3. MRI of vascular permeability in tumor-bearing mice   *months 12 –36*
   In progress
   The initial results on tumor perfusion and water content with and without contrast were reported in the progress report for year 1. Current studies are centered on the effect of peptides on tumor vessel permeability, blood flow and the transport of macromolecules. These are evaluated using contrast agents with different molecular weights such as DTPA conjugated to IgG or albumin and labeled with a mixture of ^{153}Gd and non-radioactive Gd isotope. The development of data analyses of tumor vessel permeability is in progress.

The Objective 3 is primarily concerned with the therapeutic studies (To conduct experimental therapy studies with ^{131}I-B72.3 RIT and in combination ^{131}I-B72.3 with peptide in human prostate cancer model) and the following specific tasks are associated with this Objective:

1. RIT of prostate cancer in tumor-bearing mice   *months 12 –36*
   In progress
   This is an ongoing task in years 2 and 3 of this project and overlaps with the next task 2 in this Objective.

2. Biodistributions and termination of therapy protocols   *months 12 –36*
   In progress
   All therapy protocols are terminated when the tumor size in control groups reaches about 1g. We continue to evaluate antibody retention and tumor vascular changes in necropsied tumor samples. When possible, i.e., sufficient recovery of radiolabeled material in blood, a determination of the immunoreactivity of recovered radioactive species and levels of TAG-72 in treated and non-treated tumors is also performed.

In the final Objective 4 a comparison of the efficacy of ^{131}I-labeled versus ^{90}Y-antibodies in a pepotide-augmented RIT protocol will be evaluated. These studies will begin in the next few months. The experimental evidence from carcinoma xenografts indicate that ^{90}Y may be a superior choice as a therapeutic radioisotope. To date, there are no studies to confirm this in prostate cancer xenografts. There are also no studies on the effect of biological response modifiers, e.g., our C5aAP on the outcome of ^{90}Y-RIT. We will investigate this question in the last three months

of these studies. The end points will be the same as for ^{131}I-RIT, i.e., tumor growth delay, VP changes and the general health of the treated animal.

Results

In Vitro Studies

Several reports indicate that the C5a complement may play an important role in apoptosis (Riedemann et al, 2002; Perianayagam et al., 2002). The likelihood that C5aAP has a direct effect on apoptosis of prostate carcinoma cells was investigated in vitro. Because C5AP binds to C5aR the expression of C5aR on the PC-3, DU-145 and LNCaP cells was evaluated by flow cytometry (the results of these experiments were detailed in the Progress Report for year 1). To measure the cytotoxicity, if any, of the C5aAP peptides on these cells, different concentrations of C5aAP were added to the growth medium and proliferative or cytotoxic effect were measured using either a clonogenic assay or the CellTiter 96® cell proliferation assay. The effects of C5aAP on the radiosensitivity of the prostate adenocarcinoma cells were also measured. Only at high concentrations of the C5aAP peptides, the fraction of metabolically active cells was diminished. For example, after a 48-h continuous exposure to C5aAP, approximately 30% of PC3 cells did not survive the treatment. However, after 120 hours the cells recovered and there were no detectable statistical differences between irradiated cells treated with C5aAP and cells "sham" treated with PBS. Combination of irradiation and C5aAP treatment does not seem to produce any synergistic or additive effects. To establish if C5aAP influence the cell cycle and apoptosis cells were seeded into 25 cm^2 flasks (5 x 10^5 cell/ flask) with different concentration of C5aAP. Then, 24 hrs after seeding, some flasks were irradiated at 1 Gy or 6 Gy. After 48 hrs to allow for repair/death, the cells were fixed with 70 % ethanol and stained with the Telford reagent overnight at 4°C to allow maximal intercalation of propidium iodide. The cells were analyzed by flow cytometry. There were statistical differences between cells that were irradiated and the ones that were not, however these changes were not dependent on the presence or absence of C5aAP in the growth medium. Also, there were not statistically differences in apoptotic fractions after C5aAP treatment.

Our experimental results indicate that the macromolecular extravasation induced by the C5aAP is neutrophil-dependent (see discussion below in the section on vivo studies). Other possible mechanisms attribute the macromolecular efflux to extracellular liberation of neutrophils-release products such as oxygen radicals that can undermine vascular integrity by direct or indirect actions on endothelial cells or other components of vascular walls, glycocalyx, basement membrane, etc. The production of reactive oxygen species by C5aAP interaction with neutrophils is of particular interest to RIT. The generation of superoxides of hydrogen peroxide may be responsible for the enhanced responses of tumors to RIT. Superoxide and its derived active oxygen species are believed to be responsible for the polymorphonuclear leukocyte (PMN)-mediated tumoricidal activity of the intraperitoneal OK-432, a biological response modifier (Yoshikawa et al., 1995). The role of C5aAP in the production of the hydrogen peroxide and other reactive species was studied in vitro in cancer cells and in vivo and ex vivo in blood cells.

The production of hydrogen peroxide was measured in the DCF assay by the oxidation of the nonfluorescent compound 2',7' –dichlorofluorescein (DCFH) to the fluorescent compound 2',7' - dichlorofluorescein (DCF). The nonpolar and nonfluorescent form of DCFH is 2',7' -
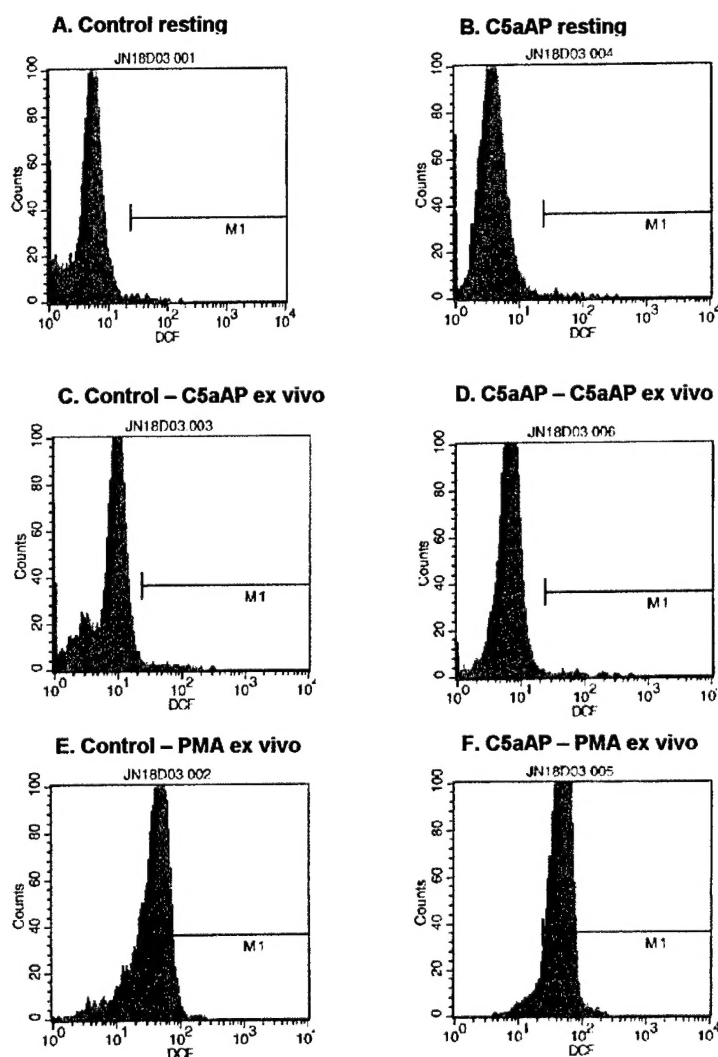
Figure 1. Flow cytometry evaluation of hydrogen peroxide production by neutrophils.

dichlorofluorescein diacetate (DCFH-DA). DCFH-DA readily diffuses across the cytoplasmic membrane of PMNs and is trapped inside the cell by the hydrolysis of acetyl groups by cytoplasmic carboxylases to the nonfluorescent and charged DCFH. Stimulation of the oxidative burst of DCFH-labeled phagocytes can be readily detected by flow cytometry. The advantage of this assay is the ability to evaluate PMNs in whole blood samples, thus avoiding the stimulatory effects often encountered in many PMN purification procedures.

EDTA-anticoagulated whole blood (0.5 ml) was obtained from mice treated with C5aAP 3 h before blood collection and from normal control mice injected with PBS also 3 h before blood collection. Each blood sample was analyzed separately and also blood collected from 3 mice in each group was pooled and the result compared to individual samples. Phorbol 12-myristate, 13-acetate (PMA) was used for ex vivo stimulation (positive controls). Red blood cell lysing buffer was added to each sample. White blood cells were pelleted by centrifugation at 1,000xg for 1 min and the cell pellet was washed with PBS and resuspended in 0.1 ml of PBS. An aliquot of 0.1 ml 10 µM DCFH-A was added to each tube, vortexed, and incubated for 15 min at 37°C at which time 0.7 ml of catalase solution was added. PMA working solution (0.1 mL of 1 µg/ml in PBS) was added to each positive control samples (PMA-stimulated). The "resting" samples received 0.1 mL PBS in place of PMA. The experimental samples were stimulated with the C5aAP peptide at 100 nM. All samples were incubated for 15 min at 37°C and evaluate samples for fluorescence by flow cytometry. A standard protocol for whole blood samples and one-parameter fluorescence histogram acquisition was used accumulating 5,000 – 10,000 events on either gated neutrophils or monocytes or both. Gating on lymphocytes also allowed the monitoring of any possible extracellular cross-feeding of cells with hydrogen peroxide produced by stimulated phagocytes that were not degraded by catalase. The control resting sample were evaluated first and the fluorescence cursor was set to less than 2%

positive cells. This cursor setting was used for the evaluation of all remaining samples. The mean channel fluorescence of the fluorescence histogram should was recorded for all samples (Figure 1). The in vivo effects of C5aAP on the production of hydrogen peroxide were apparently lost during the cell preparation (compare Fig. 1A and 1B). However, the PMA-stimulated PMNs obtained from normal (Fig. 1E) and C5aAP-treated mice (Fig. 1F) yielded at least 95% positive cells with high mean channel fluorescence values indicating high levels of hydrogen peroxide production. The ex vivo C5aAP-stimulation also produced H_2O_2 but at much lower levels that PMA (Fig. 1C and 1D). The data is summarized in Figure 2. The cytotoxic potential of neutrophil-release oxygen products prompts further studies to clarify the precise nature of the interactions between these products, cell injury and radiation. The evaluation of superoxide levels measurements in PMNs and tumor cells in vivo and in tumor cells grown in vitro in the presence of neutrophils is in progress.
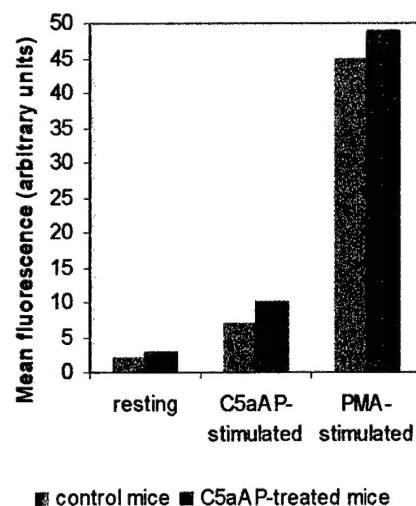

■ control mice ■ C5aAP-treated mice

Figure 2. Mean fluorescence of 2',7'-dichlorofluorescein in polymorphonuclear cells isolated from blood either control mice (green bars) or C5aAP-treated mice (red bars) either resting or stimulated ex vivo with PMA or C5aAP.

In Vivo Studies

Mechanism: The studies on the mechanism of the vascular permeability changes caused by the C5aAP peptides are completed. The interactions of the C5aAP peptides with its receptors expressed on polymorphonuclear leukocytes and the role of these interactions on the expression of the inducible nitric oxide synthase as well as the role of cyclooxygenases in vascular permeability changes induced by C5a agonist peptides are discussed in detail in the manuscript included in the Appendix A of this Progress Report (Appendix A: Role of polymorphonuclear leukocytes, nitric oxide synthase, and cyclooxygenase in vascular permeability changes induced by C5a agonist peptides. Takashi Kurizaki, Michio Abe, Sam D. Sanderson, Charles A. Enke, Janina Baranowska-Kortylewicz. *Mol. Cancer Ther.* 2004; 3(1):1–7.).

Therapy: The effects of C5aAP on radioimmunotherapy of PC3 and DU145 xenografts was evaluated in a subcutaneous tumor model in athymic mice. In all radioimmunotherapy experiments, male athymic mice (nu/nu) obtained from either NIH or Charles River (Wilmington, MA) at 4-6 weeks of age, were injected subcutaneously on the back with 1 x 107 tumor cells (DU145) or 2 mm x 2 mm PC-3 tumor sections. Tumor growth was determined using caliper measurements of the long and short axis of each tumor and the tumor volume was calculated as follows: Volume = π x (mm, short axis)^2 × (mm, long axis)/6. Three to four week after the tumor implantation the mice were assigned into groups (n=10) in such a way as to give a similar tumor size distribution in all treatment groups. The average tumor volume was maintained in all groups at 400 mm^3 on the day of therapy. Control mice were injected IV with PBS; the treatment groups included mice treated with a single dose of 250 µCi [131]ICC49; mice treated with 200 µg C5aAP peptide; and finally mice treated with a combination of 250 µCi [131]ICC49 and 200 µg C5aAP peptide. The peptide was

injected 3 hours before the ^{131}ICC49 injection. Body weights and tumor sizes were monitored twice a week. Mice were observed until tumors exceeded 10% of the total body weight or until the tumors began to ulcerate through the skin or if mice lost 20% or more of their original weight, at that time the animals were removed from the group and euthanized. The survival fraction of each treatment group was evaluated according to the method of Kaplan and Meier. The survival curves were compared and p values were generated using the logrank test. The GraphPad Prism software (GraphPad Software Inc., San Diego, CA) was used for these analyses. The survival curves for PC3 tumors built based on the quadrupling of tumor volume are shown in Fig. 3 and in Table 1.
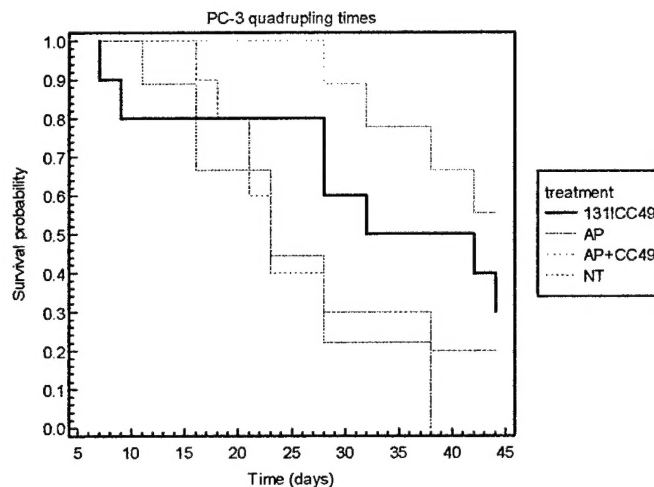

Figure 3. Survival curves of mice bearing PC3 prostate adenocarcinoma xenografts treated with 0.25 mCi ^{131}ICC49 monoclonal antibodies (heavy solid line); with C5aAP (light solid line); a combination of 0.25 mCi ^{131}ICC49 and C5aAP (short dashed line) and untreated (long dashed line).
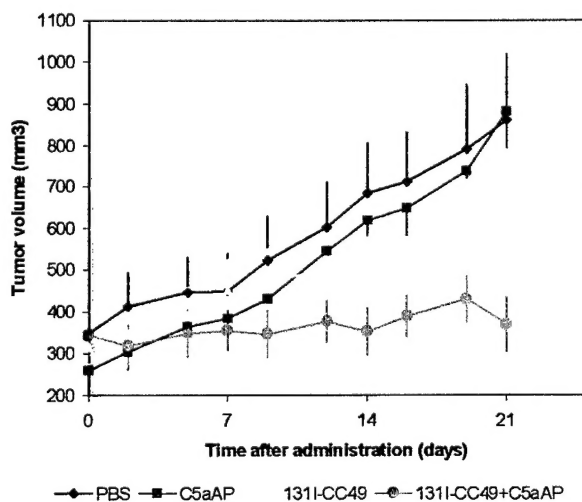

Figure 4. Changes of DU145 tumor volumes after treatment with PBS (navy); C5aAP (purple); ^{131}ICC49 (yellow), and ^{131}ICC49 + C5aAP (light blue).

A similar treatment was applied to mice bearing DU145 xenografts. The data is summarized in Figure 4 below. Statistical analyses of the therapy data after treatment of DU145-bearing mice with either ^{131}ICC49 alone or in combination with the C5aAP peptide is shown in Table 2. Statistically significant differences between various treatment and control groups emerge as soon as 14 days after the treatment and persist until the termination of the therapy experiments due to the uncontrolled growth of the untreated tumors. The comparison was made using an unpaired t-test with Welch correction for comparison of treatment groups with controls. The Kaplan-Meier analyses of these data gives results similar to these reported for PC3 tumor with P < 0.02 (analyses not shown). Of

Table 2. Summary of the Kaplan-Meier analyses of the tumor quadrupling times survival curves.

Treatment	[131]ICC49	C5aAP	[131]ICC49 +C5aAP	No treatment
Evaluated sample size	10	9	10	10
Median survival	37 days	23 days	Not determined	23 days

Chi-square = 8.9448; Significance P = 0.0300

the two MAb selected for preclinical RIT studies, CC49 appears to have more favorable uptake in PC3 and DU145 tumors. CC49 is a second generation MAb that recognizes TAG-27, the same antigen as B72.3. Similarly to B72.3, CC49 also has a significant reactivity with over 85% of adenocarcinomas including prostate cancer and only a minimal reactivity with normal tissues, but its TAG-72-specificity and affinity are significantly higher that B72.3. Both antibodies when labeled with therapeutic radioisotopes arrested or significantly delayed growth of subcutaneous adenocarcinomas in mice in a dose-dependent manner.

Because the advantages of the adjuvant treatment are more apparent in a condition where the degree of response to RIT is less than optimal, we have used doses of 0.25 mCi [131]IB72.3 in LNCaP studies reported previously. This approach also worked well in the pilot experiments in the LS174T tumors. However, when the uptake studies were performed in PC3 or DU145 prostate adenocarcinoma xenografts, the accumulation of [125]IB72.3 in tumors was approximately five-to-ten times lower than in LS174T xenografts depending on the tumor size. For this reason subsequent therapy studies in these two tumor models were done with ^{131}I-CC49. As reported previously, based on the biodistribution studies in athymic mice bearing PC3 tumors, the accumulation of [125]ICC49 in PC3 tumors is still lower than the uptake of this antibody in LS174, but significantly higher compared to [125]IB72.3. Also, the effect of C5aAP on the tumor uptake of [125]ICC49 is more pronounced in the case of CC49 than B72.3. The overall increase in the accumulation of [125]ICC49 in PC3 tumors in response to 0.3 mg C5aAP is >80% compared to a

Table 1. Statistical analyses of the therapy data after treatment of DU145-bearing mice with either [131]ICC49 alone or in combination with the C5aAP peptide (P values). The comparison was made using an unpaired t-test with Welch correction for comparison of treatment groups with controls.

| Treatment	day 0	day 5	day 7	day 9	day 12	day 14	day 16	day 19	day 21	day 23
C5aAP vs PBS	0.261	0.384	0.524	0.464	0.689	0.683	0.683	0.783	0.930	0.741
[131]ICC49 vs PBS	0.695	0.595	0.980	0.668	0.471	0.192	0.137	0.347	0.427	0.475
[131]ICC49+C5aAP vs PBS	0.949	0.315	0.346	0.141	0.061	**0.016**	**0.017**	**0.031**	**0.024**	**0.074**
[131]ICC49 vs C5aAP	0.318	0.744	0.433	0.741	0.773	0.367	0.264	0.438	0.310	0.185
[131]ICC49+C5aAP vs C5aAP	0.243	0.822	0.676	0.331	0.138	**0.034**	**0.033**	**0.019**	**0.008**	**0.012**
[131]ICC49+C5aAP vs [131]ICC49	0.723	0.616	0.256	0.237	0.183	0.130	0.238	**0.049**	**0.014**	**0.033**
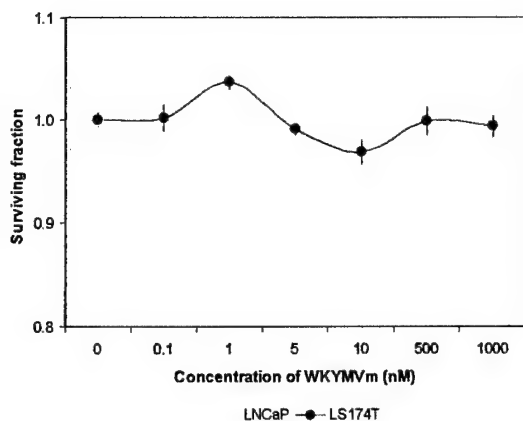
Figure 5. Surviving fraction of LNCaP cells (yellow squares) and LS174T cells (purple circles) after treatment with increasing concentrations of WKYMVm peptide.

little over 20% when ^{125}IB72.3 is injected. From the standpoint of RIT, low levels of expression of TAG-72 in PC3 tumors and consequently, the low uptake of ^{125}ICC49 in these xenografts are the "worst case" scenarios. Good responses to RIT with a combination ^{131}ICC49 plus C5aAP in this tumor model, give confidence that the C5aAP-augmented approach to RIT in other prostate cancer models and in clinical situations will also be successful. C5aAP has a noteworthy effect on the clearance of ^{125}ICC49 from the systemic circulation during the first 2 h after the C5aAP dosing. It appears that within <15 min post-injection, CC49 already escaped the vascular spaces. The subsequent return of the radioactivity into the blood requires approximately 60 min until the steady state is achieved. After 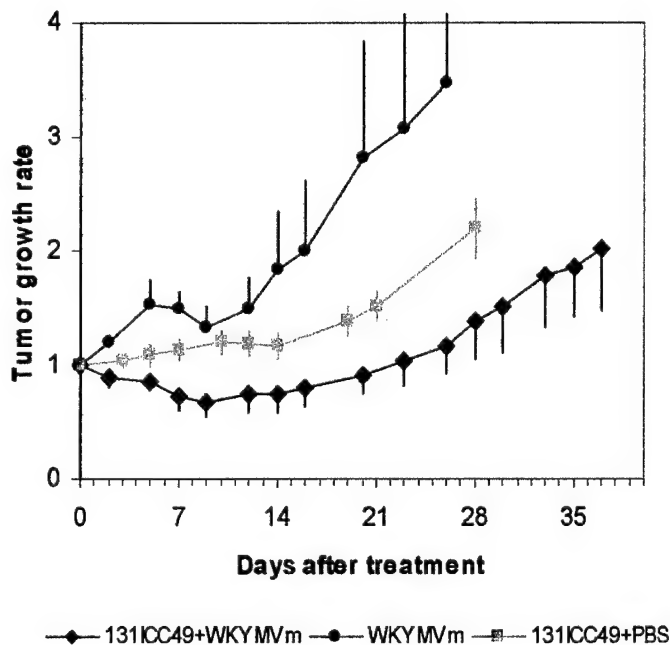this time, the elimination phase and the circulating levels of ^{125}ICC49 in mice treated with C5aAP and PBS (controls) are virtually identical.

WKYMVm-Augmented RIT of LNCaP Tumors

Based on the data from the therapy studies and the ex vivo studies on the production of the reactive oxygen species, it became apparent that in addition to the changes in the vascular permeability of tumor blood vessels and consequently improved uptake of the radiolabeled antibodies, the improvement of the RIT outcome includes also other factors. To test this hypothesis a new peptide was included in the therapy studies. Prior to the therapy studies, the cytotoxicity of WKYMVm peptide was tested in vitro (Figure 5). WKYMVm stimulates leukocytes via a unique cell-surface receptor distinct from the receptors for C5a and various other ligands that stimulate human leukocytes (Seo et al., 1997, 1998). On a functional basis, this peptide stimulates superoxide generation and bacteria killing activity in human neutrophils and monocytes

Figure 6. LNCaP tumor growth curves after indicated treatments. ^{131}ICC49 (0.25 mCi) was administered IV on day 0. Symbols indicate averages, bars indicate standard errors; n = 10.

through the interaction with formyl peptide receptor-like 1 (FPRL1). The vascular permeability is also affected by this peptide indicating that the generation of the reactive oxygen species contributes to these changes; however, the downstream signaling pathways for this peptide are distinctly different than for C5aAP peptides. Neutrophils perform a critical role in innate immune responses, including extravasation from the peripheral blood stream, migration into an infected area, and the generation of reactive oxygen species such as superoxide (Baggiolini et al., 1993; Bokoch, 1995).

Further mechanistic studies on the chemotactic migration and superoxide generation in human neutrophils as well as the activation of the respiratory burst system are in progress in vitro and in tumor-bearing and normal mice. The data gathered to date is insufficient to derive any final conclusions. However, based on the therapy data it appears that the activation of neutrophils by two

Figure 7. Changes in body weight in mice treated with WKYMVm peptide alone (red circles) or with 131ICC49 monoclonal antibody (0.25 mCi/mouse) and WKYMVm peptide (navy squares).

different ligands i.e., C5aAP peptides and WKYMVm can induce differential cellular signaling and unique functional consequences in human neutrophils. Experiments involving both types of peptides in combination with RIT are planned to begin in the next few months. The pilot therapy studies in LNCaP-bearing mice were completed and the data is shown in Figure 6. All mice received SSKI in their drinking water 3 days before the treatment with radiolabeled antibodies. Control mice also received SSKI to assure identical handling of animals. Radioiodinated [131]ICC49 was administered at a concentration of 0.25 mCi/0.2 mL PBS/mouse. Mice receiving a combination of peptide and antibodies were injected with 0.1 mg WKYMVm peptide in mixed with the dose of 0.25 mCi [131]ICC49 in 0.2 mL PBS. The control mice treated with only peptide received 0.2 mg peptide in 0.2 mL PBS. The arrest of tumor growth in a combination RIT + WKYMVm peptide group is remarkable. Three days after the treatment there is a statistically significant difference between the combination therapy group and the controls. The concern is the
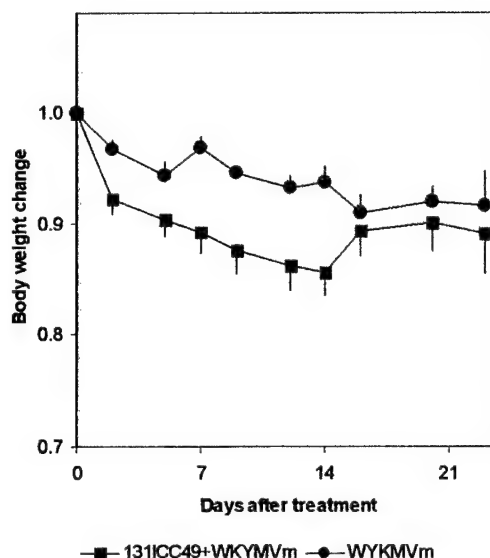
Table 2. Statistical analyses of the therapy data after treatment of LNCaP-bearing mice with either [131]ICC49 alone or in combination with the peptide WKYMVm. The comparison was made using an unpaired t-test with Welch correction for comparison of treatment groups ([131]ICC49+WKYMVm) with controls ([131]ICC49+PBS; WKYMVm alone).

Treatment	day 3	day 5	day 9	day 14	day 20	day 23
[131]ICC49+WKYMVm vs [131]ICC49+PBS	0.178	0.110	0.006	0.039	0.008	0.006
[131]ICC49+WKYMVm vs WKYMVm	0.017	0.008	0.009	0.034	0.053	0.030

loss of the body weight (Figure 7) not observed previously with the C5a-based peptides. Mice do not appear to fully recover their weight even three weeks after the treatment. The underlying causes of the weight losses are at the moment unknown and will be further explored when the benefits of including this peptide in PC3 and DU145 models are confirmed. Additional studies that also include radiotherapy using the external beam irradiation combined with the peptide treatment to confirm the role of the reactive oxygen species are also planned.

Key Research Accomplishments to Date
✓ Beneficial effects of the C5aAP peptides inclusion in the radioimmunotherapy protocols were confirmed in three prostate adenocarcinoma models: LNCaP; DU145 and PC3.
✓ Determined the expression of C5a receptors and effect of a peptide agonist of human C5a complement on the metabolic activities of in vitro grown prostate adenocarcinoma cells.
✓ Functions of the C5aAP peptides in RIT and the basic mechanism of their action were established.
✓ The production of reactive oxygen species was identified as a possible additional factor in the improvement of the RIT results when combined with the C5aAP treatment.
✓ New peptide WKYMVm that increases vascular permeability and generates superoxide was used in a RIT protocol and was shown to greatly improve the outcome of RIT indicating that the synergy/additive effects observed with C5aAP may be at least partially a result of the interaction of radiation with in situ generated reactive oxygen species.
✓ Using noninvasive MRI techniques, determined the C5aAP-induced changes in tumor perfusion in the experimental model of human prostate adenocarcinoma. The MRI studies of the changes in the vascular permeability are in progress.

Reportable Outcomes
Role of polymorphonuclear leukocytes, nitric oxide synthase, and cyclooxygenase in vascular permeability changes induced by C5a agonist peptides. Takashi Kurizaki, Michio Abe, Sam D. Sanderson, Charles A. Enke, Janina Baranowska-Kortylewicz. Mol. Cancer Ther. 2004; 3(1):1–7.

Conclusion
✓ The inclusion of C5aAP in RIT improves the outcome of RIT of the experimental prostate adenocarcinoma grown as a subcutaneous xenografts in athymic mice. Tumors treated with C5aAP and [131]ICC49 antibodies grows significantly slower that tumors treated with [131]ICC49.
✓ C5a Receptors are expressed on some prostate cancer cells and may have an effect on the tumor growth and response to RIT.
✓ Increased levels of tumor oxygenation in response to stimulation of neutrophils with peptides appear to radiosensitize tumors to radioimmunotherapy.

Abbreviations:
C5aAP	response-selective peptide agonist on the human C5a complement
CC49	second generation monoclonal antibody that recognizes TAG72 in most of human adenocarcinomas
B72.3	precursor monoclonal antibody of CC49
nM	concentration: nanomole/liter

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APPENDIX A

Role of polymorphonuclear leukocytes, nitric oxide synthase, and cyclooxygenase in vascular permeability changes induced by C5a agonist peptides. Takashi Kurizaki, Michio Abe, Sam D. Sanderson, Charles A. Enke, Janina Baranowska-Kortylewicz. *Mol. Cancer Ther*. 2004; 3(1):1–7.

Role of polymorphonuclear leukocytes, nitric oxide synthase, and cyclooxygenase in vascular permeability changes induced by C5a agonist peptides

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Abstract

Q1 Tumor responses to radioimmunotherapy combined with peptide agonists of human C5a anaphylatoxin such as GCGYSFKPMPLaR (C5aAP) are several-fold better than responses to radioimmunotherapy alone. The enhanced tumor vascular permeability (VP) is the key factor responsible for this improvement. These studies were designed to identify the sequence of events leading to the improved extravasation of immunoglobulin in response to C5aAP. The VP changes were measured in mice after administration of C5aAP alongside of various mediators. The depletion of circulating polymorphonuclear neutrophils (PMN) in mice abolished the C5aAP-induced VP increase. Blocking of P-selectin also returned VP to its basal levels after the C5aAP treatment, indicating that C5aAP-induced VP changes are initiated by interactions of C5aAP with PMNs. Aminoguanidine, an inducible nitric oxide synthase (NOS) inhibitor, given before C5aAP returned VP to control levels. N^G-nitro-L-arginine methyl ester, a nonselective NOS inhibitor, had a marginal effect on the activity of C5aAP. Indomethacin, a nonselective cyclooxygenase inhibitor, suppressed C5aAP-induced increases in VP, whereas N-(2-cyclohexyloxy-4-nitrophenyl)-methanesulfonamide, a selective cyclooxygenase-2 inhibitor, was active only at high doses. While C5aAP given i.p. did not alter tumor uptake of ^{125}I-B72.3, the i.v. administration resulted in ~40% increase, confirming the prerequisite interaction of C5aAP with PMNs. The sequence leading to the increased VP appears to be initiated by the interaction of C5aAP with C5a receptor expressed on PMNs followed by binding to endothelial cells of blood vessels. The interaction with P-selectin is responsible for the initiation of the nitric oxide cascade as evidenced by inducible NOS activation. Additionally, prostaglandins are required for expression of the full magnitude of the C5aAP activities. (Mol Cancer Ther. 2004;3(1):1–7)

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Introduction

C5a, a small activation fragment of the complement C5 protein induced by either classical or alternative pathway, is a potent proinflammatory mediator (1). It binds specifically to its receptor, C5a receptor (C5aR; CD88), from the superfamily of G protein-coupled receptors expressed on a variety of cells of myeloid and nonmyeloid origins (2, 3). On binding to CD88, C5a evokes anaphylactic and chemotactic (attractant) responses, which mediate contraction of smooth muscles, enhance vascular permeability (VP), and promote leukocyte functions such as directed chemotaxis, degranulation, mediator release, and production of superoxide anions. Of special interest to therapy of solid tumors is the ability of C5a to profoundly increase permeability of blood vessels (4), resulting in facilitated transport of macromolecular drugs into the solid tumor. However, systemic administration of C5a is contraindicated because of the possible adverse effects. A panel of C5a agonist peptides was developed to address some of these deficiencies. Based on the structure-activity study of the COOH-terminal domain of C5a, peptide agonists with varied C5aR affinities and diverse selectivity to cells expressing C5aR were synthesized (5).

One of the conformationally biased agonist peptides of human C5a, YSFKPMPLaR (C5aAP), has been reported to increase VP in the skin of guinea pig (6). Studies from our laboratories revealed that C5aAP and its GCG-modified analogue, GCGYSFKPMPLaR, improve the outcome of radioimmunotherapy (RIT) in the experimental human colorectal cancer xenografts in athymic mice by the induction of transient increases of VP (7). However, the mechanism of C5aAP-induced changes in VP and the events leading to the synergy between C5aAP and RIT are unclear. C5aAP alone has no effect on the tumor growth; therefore, the recruitment of proinflammatory cells into the tumor site is an unlikely reason for this augmented effect. Because C5a-primed polymorphonuclear neutrophils (PMNs) induce hyperpermeability and phosphorylation of adherens junction proteins in endothelial cells (8, 9) and in a similar manner C5aAP induces transient hypotension and neutropenia in rats (10), a hypothesis was put forth that C5aAP interactions with PMNs can initiate a series of events leading to the enhancement of VP. Additionally, an effort was made to identify mediators responsible for the amplification of this initial stimulus. Some of the known mechanisms of amplification include local activation of

precursors for expanding proinflammatory cascades of plasma proteins, up-regulation by early inflammatory mediators of cell surface molecules that promote the recruitment of leukocytes (*e.g.*, adhesion molecules), and rapid leukocyte-selective expression of inducible genes, the products of which are proinflammatory [*e.g.*, cyclooxygenase (COX) 2, the first enzyme of arachidonic acid cascade, and nitric oxide (NO) synthase (NOS)]. Inducible NOS (iNOS) has been detected in activated macrophages, PMNs, and endothelium. Moreover, NO and prostaglandins are known mediators of VP changes induced by lipopolysaccharide (11) and other VP factors (12). In these studies, the *in vivo* effect of C5aAP binding to C5aR expressed on PMNs, the interactions of thus primed PMNs with P-selectin, and the role of NOS and COX are considered.

Materials and Methods

Peptides, Antibodies, and Reagents

C5aAP was synthesized by a standard solid-phase method, purified, and characterized according to the previously described procedures (5). Human recombinant complement C5a peptide (rC5a) was purchased from Sigma Chemical Co. (St. Louis, MO). Human cloned C5aR was purchased from BioSignal (Montreal, Canada).

Mouse and rat IgG, N^G-nitro-L-arginine methyl ester (L-NAME), aminoguanidine hemisulfate salt (AG), and indomethacin were from Sigma. N-(2-cyclohexyloxy-4-nitrophenyl)-methanesulfonamide (NS-398) was purchased from Biomol (Plymouth Meeting, PA). Antimouse granulocytes rat IgG2b (clone RB6-8C5) and rat antimouse P-selectin (CD62P) IgG1 (clone RB40.34) were from Leinco Technologies (St. Louis, MO) and Research Diagnostics (Flanders, NJ), respectively.

C5aR Binding Assay

Q2 Ten micrograms of rC5a were labeled with 1 mCi of $Na^{125}I$ using the Iodo-Gen method and purified on a desalting column (Econo-Pac 10DG, Bio-Rad, Hercules CA) equilibrated with 0.01 M phosphate buffer, 0.0027 M KCl, 0.137 M NaCl (pH 7.4; PBS) at room temperature. Human cloned C5aR from Chinese hamster ovary (CHO) cells was incubated with ^{125}I-rC5a at a final concentration of 0.05 nM and various concentrations of unlabeled ligands for 60 min at room temperature in the incubation buffer composed of 25 mM HEPES (pH 7.4) with 2 mM $CaCl_2$, 1 mM $MgCl_2$, and 0.2% BSA. The incubation mixture was filtered through GF/C filter (Whatman, England) presoaked in 0.3% polyethyleneimine in the incubation buffer. Filters were washed 9 times with ice-cold 10 mM HEPES (pH 7.4), 0.5 M NaCl. The radioactivity associated with filters was measured in the gamma counter (receptor-bound ^{125}I-rC5a).

General Procedure for the Assessment of VP Changes

Athymic female mice, 4–6 weeks old, were used to measure cutaneous VP induced by C5aAP. Murine IgG was iodinated with $Na^{125}I$ (specific activity ~2–3 mCi/mg) using the Iodo-Gen method and purified on a desalting column equilibrated with PBS. ^{125}I-IgG was given i.v. via a

tail vein with or without C5aAP in a total volume of 0.2 ml PBS. C5aAP doses were 20 mg/kg in the PMN depletion study and 5 mg/kg in all other studies. Thirty minutes after ^{125}I-IgG administration, mice were euthanized, their blood and ears were collected and weighed, and their radioactive content was determined in a gamma counter.

PMN Depletion

Mice were treated i.p. with antigranulocyte monoclonal antibody RB6-8C5 (anti-Ly-6G) at a concentration of 0.2 mg/mouse (10 mg/kg) in 0.4 ml PBS 26 h before ^{125}I-IgG administration. Control mice were treated with nonspecific rat IgG (instead of RB6-8C5) also at 0.2 mg/mouse in 0.4 ml PBS. Blood samples for leukocyte counting were taken from tail 2 h before ^{125}I-IgG administration. Blood smears were stained with Wright stain for the differential counting.

Anti-P-selectin Pretreatment

Mice received i.v. 0.06 mg/mouse (3 mg/kg) doses of the antimouse P-selectin monoclonal antibody RB40.34 in 0.1 ml PBS 5 min before C5aAP and ^{125}I-IgG administration. This time point was selected based on previous reports (13). Control mice were treated with nonspecific rat IgG (3 mg/kg in 0.1 ml PBS).

Mediators

To investigate the role of NOS in C5aAP-induced VP changes, two inhibitors of NOS were used: L-NAME, a nonselective, general NOS inhibitor (14, 15), and AG, a selective inhibitor of iNOS (16). Inhibitors were dissolved in PBS at 2 mg/ml L-NAME and 4 mg/ml AG. I.v. doses of **Q3** 10 mg/kg L-NAME or 20 mg/kg AG both in 0.1 ml PBS were given via a tail vein 5 min before ^{125}I-IgG administration. Mice were randomly divided into six groups treated as follows: (1) two control groups: sham injections of 0.1 ml PBS instead of inhibitor followed 5 min later by i.v. ^{125}I-IgG either alone or in combination with 0.1 mg C5aAP; (2) two L-NAME groups: i.v. injection of L-NAME at the dose of 0.2 mg/mouse in 0.1 ml PBS followed 5 min later by i.v. ^{125}I-IgG either alone or in combination with 0.1 mg C5aAP; and (3) two AG groups: i.v. dose of AG at 0.4 mg/mouse in 0.1 ml PBS followed 5 min later by i.v. ^{125}I-IgG either alone or in combination with 0.1 mg C5aAP.

The involvement of COX in C5aAP-mediated changes of VP was probed using indomethacin, a nonselective COX inhibitor, and NS-398, a selective COX2 inhibitor (17). COX inhibitors were dissolved in 25% propylene glycol (PG) and given i.p. 35 min before ^{125}I-IgG at a single dose of 0.1 mg/mouse (5 mg/kg) for indomethacin and at two levels of 0.002 mg/mouse (0.1 mg/kg) and 0.1 mg/mouse (5 mg/kg) for NS-398. All COX inhibitors were given in 0.2 ml 25% PG. Control mice were injected with vehicle alone using the same timing of events. Mice were divided into six groups as indicated for NOS inhibitors.

Tumor Uptake

Groups of mice with human colorectal adenocarcinoma LS174T xenografts received either an i.v. or an i.p. dose of C5aAP in 0.2 ml of 0.1% albumin in PBS. Three hours later, an i.v. or i.p. dose of ^{125}I-B72.3, a monoclonal mouse

antibody specific to Tag-72, an antigen expressed by *in vivo* grown LS174T, in 0.1% albumin in PBS was given. Control mice were treated with ^{125}I-B72.3 alone and a sham injection of PBS. Twenty-four hours later, mice were euthanized and necropsy was performed. Blood, lung, heart, spleen, liver, kidney, uterus, muscle, stomach, small intestine, large intestine, skin, and tumor were harvested. Radioactivity and weight of aforementioned tissues were determined.

Statistical Analysis

All results are expressed as means ± SD unless otherwise specified. Statistical significance was determined using the unpaired, two-tailed Student's t test.

Results

C5aR Binding Assay

Affinities of C5aAP and rC5a to human cloned C5aR from CHO cells were measured in a competitive binding assay. Fig. 1 shows a typical binding profile. ^{125}I-rC5a binding to C5aR is inhibited by C5aAP with IC_{50} of 1.67 ± 0.58 nM compared with 0.33 ± 0.10 nM for rC5a (0.05 > P > 0.02). C5aAP is ~5 times less effective in competing for the binding sites with ^{125}I-rC5a than rC5a. Previous reports place this figure at ~0.2% in a binding assay conducted on isolated PMNs and peritoneal macrophages (18).

The cutaneous VP changes were measured *in vivo* using the uptake of ^{125}I-IgG in skin of athymic mice and compared with the skin uptake of ^{125}I-IgG in the absence of C5aAP as a control. The nonspecific scrambled version of the C5aAP peptide was not included as a control based on the results of structure-activity studies of a series of peptides derived from the C5a complement (6). These studies indicted that only peptides, which obey rigid structural requirements, can modify the VP. Moreover, VP changes have been shown to be dependent on the circulating C5aAP concentration (7), indicating that specific interactions of C5aAP with the receptor are required.
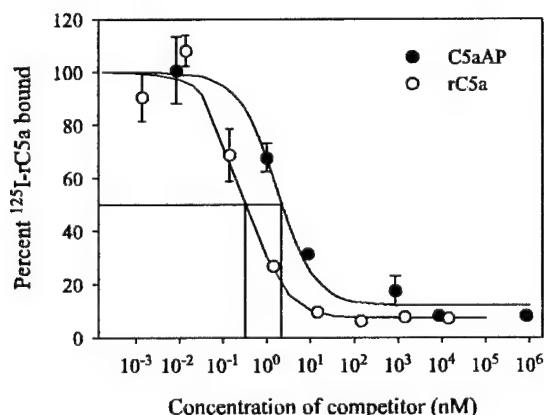

Figure 1. Competition binding profile of human rC5a, ^{125}I-rC5a, to the cloned human C5aR from CHO cells in the presence of increasing concentrations of rC5a (*open circles*) and C5aAP (*closed circles*). *Points*, mean of duplicate determinations; *bars*, SD.

Effect of PMN Depletion on C5aAP-induced VP

To confirm that the initial stimulus originates from the interaction of C5aAP with blood components, studies of the C5aAP action were conducted in PMN-depleted mice. Antigranulocyte monoclonal antibody RB6-8C5 (anti-Ly-6G), a rat antimouse IgG2b, which selectively binds and depletes mouse neutrophils and eosinophils but not lymphocytes or macrophages, was used to deplete PMNs. This antibody after an i.p. dose of 0.2 mg/mouse produces within 24 h of administration severe peripheral neutropenia persisting for up to 96 h (19, 20). A dose of anti-PMN antibodies (10 mg/kg) was injected i.p. 26 h before the administration of C5aAP and ^{125}I-IgG. A differential count of leukocytes in peripheral blood smears was 3879 ± 615/μl in control mice treated with nonspecific rat IgG (n = 8). This number in PMN-depleted mice (n = 8) treated with RB6-8C5 was 935 ± 442/μl (P < 0.001). Both counts were taken 24 h after i.p. administration of RB6-8C5. Two hours later, C5aAP and ^{125}I-IgG were injected simultaneously into the tail vein and biodistribution was conducted 30 min later. Blood and skin (ears) were collected to measure cutaneous VP (Fig. 2). No changes were detected at a dose of 0.1 mg C5aAP/mouse (5 mg/kg) in either control or PMN-depleted mice (data not shown) almost certainly because the i.p. stimulation associated with the administration of anti-PMN antibodies and control rat IgG followed by the blood collection resulted in a proinflammatory reaction sufficient to mask the effect of a low dose of C5aAP. To distinguish this response from the C5aAP-induced VP changes, a higher dose of C5aAP (20 mg/kg) was used in this assay. PMN depletion had no effect on the basal level of VP; however, it resulted in a significant inhibition of C5aAP-induced VP increases (P < 0.05; Fig. 2). In control mice treated with nonspecific rat IgG in place of RB6-8C5 and 0.4 mg C5aAP, the cutaneous blood levels climbed to 64.8 ± 9.2 μl blood/g skin in mice. However, on PMN depletion with RB6-8C5 antibodies, cutaneous VP remained at normal levels of 44.4 ± 4.4 μl blood/g skin after C5aAP treatment, suggesting that the initial trigger required to induce VP changes is the interaction of C5aAP with circulating PMNs.

Anti-P-selectin Pretreatment

Capture or tethering represents the first contact of PMN with the activated endothelium. P-selectin on endothelial cells is the primary adhesion molecule for capture and the initiation of rolling (21). Hence, the inquiry into the role of P-selectin in the C5aAP activity was the next step. The PMN-P-selectin interactions can be disrupted by an inhibition of or a competitive binding to P-selectin. Monoclonal antibody RB40.34 is a rat IgG1 that can block binding of mouse P-selectin to its ligand P-selectin glycoprotein ligand-1 (PSGL-1) constitutively found on all leukocytes (13, 21, 22). I.v. dosing of anti-P-selectin monoclonal antibody RB40.34, 0.06 mg/mouse in 0.1 ml PBS (0.3 mg/kg), was followed 5 min later by an i.v. administration of 0.1 mg C5aAP and ^{125}I-IgG. Two control groups received nonspecific rat IgG in place of
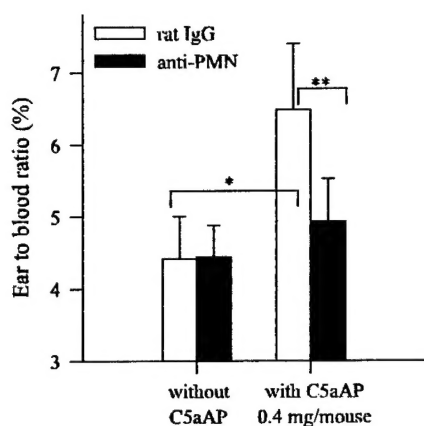
Q4

Figure 2. Effect of PMN depletion on C5aAP-induced increases in the cutaneous VP. Mice received an i.p. dose of antigranulocyte monoclonal antibody RB6-8C5, rat antimouse IgG2b (10 mg/kg in 0.4 ml PBS). Twenty-six hours later, mice were treated i.v. via a tail vein injection with ^{125}I-IgG with or without C5aAP (0.4 mg/mouse). Control mice received an i.p. dose of nonspecific rat IgG (10 mg/kg in 0.4 ml PBS) in place of RB6-8C5. *Columns*, mean ($n = 4$); *bars*, SD. *, $P < 0.05$; **, $P < 0.01$.

RB40.34 (0.06 mg/mouse) followed by either sham i.v. injection of PBS (negative control) or 0.1 mg C5aAP/mouse (positive control). Thirty minutes later, mice were euthanized and blood and ears were collected. As shown in Fig. 3, anti-P-selectin significantly inhibited C5aAP effects on VP ($P < 0.01$), indicating that the blockade of PMN rolling to the endothelial cells prevents C5aAP-induced VP increases. The cutaneous VP was 40.7 ± 4.6 ($n = 5$), 64.9 ± 13.3 ($n = 5$), and 42.7 ± 4.2 ($n = 6$) µl blood/g skin in negative control (no C5aAP treatment), positive control (C5aAP treatment), and anti-P-selectin mice (anti-PMN antibodies treatment), respectively. The P value for anti-P-selectin *versus* a positive control was $0.01 > P > 0.001$ and the P value for anti-P-selectin *versus* a negative control $P > 0.2$.

Mediators

Two potential mediators of VP were evaluated in these studies: NO and prostaglandins. To examine the role of NO, a chemical messenger produced by a family of NOS, the cutaneous VP was measured in mice treated with inhibitors of NOS. Two NOS inhibitors were tested: L-NAME, a competitive, nonselective inhibitor of all NOS (14, 15), and AG, which selectively inhibits iNOS with IC_{50} = 250 µM for iNOS and IC_{50} = 526 µM for constitutive NOS (16). Mice were treated i.v. with selected NOS inhibitors 5 min before the administration of ^{125}I-IgG alone or in combination with C5aAP. Changes in VP were assessed 30 min after administration of the radioactive tracer. The regulation of NOS activity with L-NAME had only a marginal effect on basal VP in a control group with the average increase registered at 6 µl blood/g skin ($0.1 > P > 0.05$). Similarly, in the presence of AG, basal levels of cutaneous VP remained basically unaffected (Table 1). When C5aAP was included in the treatment scheme, L-NAME, a rapidly reversible inhibitor of iNOS (15), had

no effect on VP, whereas AG, a selective iNOS inhibitor, abolished C5aAP activity and returned VP nearly to control levels ($P < 0.05$), giving a clear indication that iNOS plays a significant role in the C5aAP activities.

The synthesis of prostaglandins, important mediators of VP, is regulated by COX1 and COX2. COX1 is expressed constitutively and is present in a wide variety of cell types where it influences the physiological functions of prostaglandins, whereas COX2 is an inducible enzyme involved in those aspects of the inflammatory process that are mediated by prostaglandins. The role of these two enzymes in the C5aAP-induced VP changes was evaluated via the use of nonspecific and specific COX inhibitors in combination with C5aAP. Because the i.p. administration was required for both inhibitors, the experimental design was modified to produce a steady-state circulating concentration of the systemic inhibitor. All injections were given 35 min before the i.v. administration of ^{125}I-IgG alone or in combination with 0.1 mg C5aAP. The net effect of C5aAP in control mice was lesser after i.p. than i.v. administration of inhibitors (Table 2), indicating again that a peritoneal stimulation masks to some degree the effects of C5aAP. Pretreatment with indomethacin (5 mg/kg), a nonselective COX inhibitor with $IC_{50} = 0.74$ µM for COX1 and 0.97 µM for COX2 (17, 23), had no effect on the basal level of VP but abolished VP gains induced by C5aAP ($P < 0.05$). In contrast, the effect of NS-398, a selective COX2 inhibitor ($IC_{50} = 1.77$ µM for COX2 and 75 µM for COX1), was equivocal. At low dose, NS-398 influenced neither the basal level nor the C5aAP-induced increase in VP. At the higher dose expected to produce steady-state levels of the inhibitor well within the range of COX1 inhibition, the normally observed C5aAP-induced VP increases were reduced to levels practically corresponding to control values. Although peritoneal stimulation appeared to interfere with COX-related VP effects, there is strong evidence that inducible COX2 is probably not involved in C5aAP
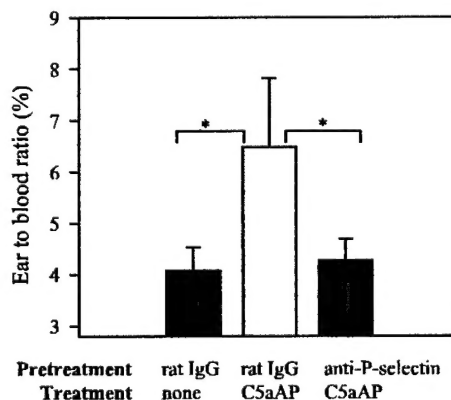

Figure 3. Results of antimouse P-selectin monoclonal antibody RB40.34 pretreatment on C5aAP-induced changes in cutaneous VP. Mice were treated i.v. with anti-P-selectin at a concentration of 3 mg/kg in 0.1 ml PBS 5 min before ^{125}I-IgG administration. *Columns*, mean ($n = 5$); *bars*, SD. *, $P < 0.01$.

Appendix

Table 1. Effects of NOS inhibitors on C5aAP-stimulated VP changes

	Vascular leakage (µl blood/g skin)	
	Without C5aAP	Treated with C5aAP
PBS control	41.0 ± 3.7	93.1 ± 28.9[a]
L-NAME (10 mg/kg)	48.7 ± 6.5	72.3 ± 23.8
AG (20 mg/kg)	41.1 ± 1.8	53.9 ± 13.1[b]

Note: Mice were treated i.v. with L-NAME (0.2 mg/mouse) or AG (0.4 mg/mouse) 5 min before ^{125}I-IgG administration. Each value represents the mean ± SD of four mice.

activity, but the activity of COX1, a constitutively expressed isoform, is clearly necessary for the C5aAP-induced VP. It follows that there is a connection between COX1-catalyzed synthesis of prostaglandin and C5aAP biological activities, although the COX1 levels remain essentially unaffected by factors responsible for COX2 induction.

Tumor Uptake

The site of injection of C5aAP (*i.e.*, a tail veil for i.v. administrations and i.p. injections) had a marked affect on the tumor uptake of ^{125}I-B72.3 and allowed to attribute, at least partially, the overall effect of C5aAP to its initial interaction with blood components. Fig. 4 shows the results of biodistribution conducted 24 h after administration of ^{125}I-B72.3 in mice bearing s.c. human colorectal adenocarcinoma. C5aAP given i.p. had no effect on tumor uptake: ^{125}I-B72.3 dose accumulated in LS174T tumors was 11.93 ± 0.68% of injected dose/g (%ID/g) in control mice treated with an i.v. dose of ^{125}I-B72.3 compared with 11.39 ± 0.52%ID/g and 11.69 ± 1.70%ID/g in mice treated with an i.v. dose of ^{125}I-B72.3 in combination with an i.p. dose of C5aAP (5 mg/kg) and with both drugs given i.p., respectively (*P* > 0.2). When C5aAP was given via a tail vein as an i.v. dose, the uptake into the tumor increased by ~40% regardless of the route of injection of ^{125}I-B72.3 and was nearly identical for i.v. (15.98 ± 0.21%ID/g) *versus* i.p. (15.05 ± 0.68%ID/g) administration of ^{125}I-B72.3. Similar to cutaneous VP changes, the full effect of C5aAP on tumor's VP appears to require an initial interaction of C5aAP with PMNs.

Table 2. Effects of COX inhibitors on C5aAP-induced VP changes

	Vascular leakage (µl blood/g skin)	
	Without C5aAP	Treated with C5aAP
25% PG (control)	38.8 ± 4.6	60.7 ± 12.4[a]
Indomethacin (5 mg/kg)	35.1 ± 2.7	45.9 ± 2.7[b]
NS-398 (0.1 mg/kg)	45.2 ± 4.8	49.2 ± 13.6
NS-398 (5 mg/kg)	ND	44.3 ± 2.1[b]

Note: Mice were treated i.p. with indomethacin or NS-398 35 min before ^{125}I-IgG administration. Each value represents the mean ± SD of four to six mice.
[a]Significantly different from basal VP (*P* < 0.05).
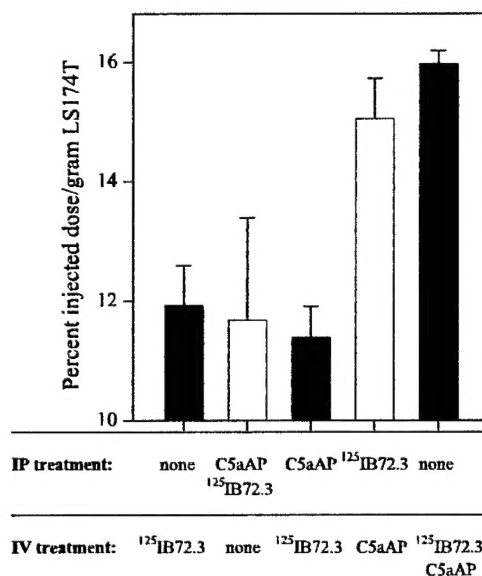[b]Significantly different from controls treated with C5aAP (*P* < 0.05).

Figure 4. Tumor uptake of ^{125}I-B72.3 in LS174T-bearing athymic mice after either an i.v. or an i.p. treatment with a single dose of C5aAP. Mice were treated with a dose of C5aAP followed 3 h later by ^{125}I-B72.3. Biodistribution was conducted at 24 h post-^{125}I-B72.3. *Columns,* mean (*n* = 4); *bars,* SD.

Discussion

Monoclonal antibodies significantly improved the targeted delivery of therapeutic radioisotopes to tumors. However, gains in selectivity are strongly counteracted by problems related to the heterogeneity of structure and physiology of solid tumors resulting in minimal radiolabeled monoclonal antibody localization at the tumor site. The accretion of radiolabeled antibodies and consequently the radiation doses deposited in tumors rely on the tumor blood flow, the tumor vascular volume, and the VP of tumor vessels to macromolecules. Methods to transiently change tumor VP have been suggested as a means to increase access of RIT to tumors (24–26). This approach was substantiated in a recent study of the C5aAP-augmented RIT in a mouse model of human colorectal adenocarcinoma (7). The improved tumor responses were attributed to the improved penetration of RIT into the tumor after the C5aAP-induced transient increase of VP. The translation of this approach to the clinic requires a comprehensive knowledge of mechanisms involved in the generation of these VP changes. Because of the inherent variability of xenograft physiology, particularly in large tumors required for the assessment of the VP changes (*e.g.*, compromised vascular structure, variable tumor vascular volumes, impaired local blood flow, variable sizes of the necrotic fraction, heterogeneous penetration of macromolecules into xenograft, etc.), the VP changes were measured in mouse skin (11, 12, 27).

The VP responses observed after C5aAP activation suggest that binding of C5aAP to the C5aR followed the magnification of this initial signal by endogenous, humoral, and cell-derived amplification systems that initiate the

Appendix

production of secondary messengers. Based on our data, the first step in the activation process involves binding of C5aAP to C5aR expressed on PMNs or endothelial cells followed by the activation of iNOS. Concurrently, C5aAP-primed leukocytes express PSGL-1, sialyl Lewis X, or a closely related oligosaccharide (28, 29). Transient interactions between P-selectin and PSGL-1 allow leukocytes to roll along the endothelium, ultimately resulting in an enhanced VP (8, 13). It has been reported that antihistamine inhibits the C5aAP-induced VP increase in guinea pig skin (6) after intradermal injection of C5aAP. In this instance, the most likely course of events involved a local response at the level of dermal mast cells, which express functional C5aR, followed by the secretion of histamine. It is doubtful however that after a systemic administration of C5aAP, scarce circulating basophils, <1% of total leukocytes, are the paramount cell population contributing to VP changes inasmuch as the PMN depletion attenuates the C5aAP-induced hyperpermeability. The absence of C5aAP-stimulated VP changes in PMN-depleted mice after the P-selectin blockade indicates that the activation of PMNs is the most plausible pathway for the C5aAP-induced VP changes.

Parallel or alternative pathways to VP enhancements involve the expression of signal amplifying mediators. Q5 INOS on activation may produce NO at the site of adhesion. It is unclear which cells are the principal source of NO (*i.e.*, PMNs or endothelial) and at which point of the amplifying cascade NO becomes a predominant factor in VP changes. It is evident however that iNOS plays a significant role in the C5aAP-induced enhancement of VP (*i.e.*, the inclusion of iNOS inhibitors in the treatment scheme abolishes all VP changes mediated by C5aAP). The existing published data are somewhat ambiguous in this context. For example, it is reported that C5a induces a dose-dependent vasodilation mediated by NO in the small intestine microvessels (30). Conversely, neutrophils exposed to C5a fail to show increases in intracellular cyclic GMP, an indicator of NO production (31). Therefore, other factors such as degranulation and release of chemical mediators such as histamine, serotonin, interleukin (IL)-1, IL-6, tumor necrosis factor, and IL-8 from mast cells, platelets, PMNs, or monocytes at the site of adhesion may also play a role in C5aAP signaling of VP modification.

The metabolic effects of C5aAP are also impaired by indomethacin, a prostanoid synthesis inhibitor. Studies on the involvement of prostanoids, histamine, and PMNs in rabbits (32) concluded that indomethacin does not alter the C5a-induced neutropenia but normalizes plasma prostanoid levels. C5aAP effects on VP are largely abolished by indomethacin, also indicating that COX products play an important role in the C5aAP-induced VP changes. However, in these studies, the coadministration of C5aAP with prostaglandin E2 (data not shown) had no measurable effect on VP. The role of COX is further complicated by the apparent resistance of C5aAP-stimulated VP increases to COX2 inhibition, suggesting that C5aAP does not regulate COX2 expression.

In conclusion, the C5aAP-induced VP increases appear to originate from the binding of this peptide to C5aR, activation of PMNs amplified by two apparently independent signals: an increased synthesis of iNOS and COX metabolic products. The net effect is an improved uptake of radiolabeled antibodies into the tumor mass, increased radiation doses, and thereby improved tumor responses to RIT.

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